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Determination of betulinic acid in mouse blood, tumor and tissue homogenates by liquid chromatography–electrospray mass spectrometry

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Abstract

A rapid and sensitive high-performance liquid chromatography–electrospray MS method has been developed to determine tissue distribution of betulinic acid in mice. The method involved deproteinization of these samples with 2.5 volumes (v/w) of acetonitrile–ethanol (1:1) and then 5 μl aliquots of the supernatant were injected onto a C_{18} reversed-phase column coupled with an electrospray MS system. The mobile phase employed isocratic elution with 80% acetonitrile for 10 min; the flow-rate was 0.7 ml/min. The column effluent was analyzed by selected ion monitoring for the negative pseudo-molecular ion of betulinic acid $[M-H]^-$ at m/z 455. The limit of detection for betulinic acid in biological samples by this method was approximately 1.4 pg and the coefficients of variation of the assay (intra- and inter-day) were generally low (below 9.1%). When athymic mice bearing human melanoma were treated with betulinic acid (500 mg/kg, i.p.), distribution was as follows: tumor, 452.2±261.2 μg/g; liver, 233.9±80.3 μg/g; lung, 74.8±63.7 μg/g; kidney, 95.8±122.8 μg/g; blood, 1.8±0.5 μg/ml. No interference was noted due to endogenous substances. These methods of analysis should be of value in future studies related to the development and characterization of betulinic acid.

Keywords: Betulinic acid

1. Introduction

Betulinic acid (Fig. 1) is found in *Ziziphus* species and is readily available from natural sources such as the bark of white birch trees [1], wherein betulin may be found in concentrations in the range of 20%. Betulinic acid inhibits carcinogenesis in the two-stage mouse skin model [2], and inhibits the growth of human melanoma carried in athymic mice [3]. With cultured melanoma cells, the mode of inhibition involves the induction of apoptosis [3]. As a result of potential antitumor activity and an ostensible lack of toxicity, betulinic acid is now being evaluated in preclinical tests. However, since betulinic acid is a poor UV chromophore, chromatographic analysis using UV detection is difficult,

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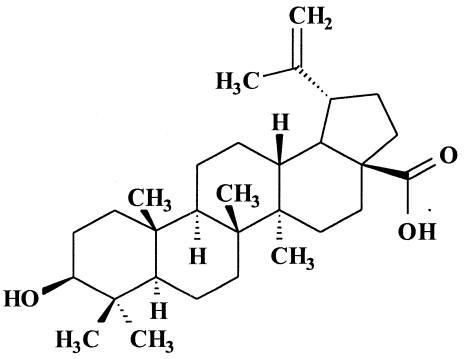

Fig. 1. Chemical structure of betulinic acid.

especially if there are substances in the sample matrix that interfere.

Although analysis of betulinic acid in *Zyziphus* species by HPLC with UV detection has been described [4], this method is not applicable to biological matrices containing only trace amounts of betulinic acid. In plant extracts, betulinic acid may be abundant (about 2–3% by weight). Therefore, it was necessary to develop simple and rapid sample preparation procedures with improved analytical recovery and greater sensitivity. Electrospray mass spectrometry is a soft ionization technique that has been shown to produce various intact ions. The method has been found useful for analysis of various molecules including peptides [5], oligonucleotides [6] and drug metabolites [7]. In this paper, we describe a HPLC–electrospray MS method for the determination of betulinic acid in mouse blood and homogenates of tumor, liver, lung or kidney.

2. Experimental

2.1. Materials

Betulinic acid was purchased from Indofine Chemical Co. (Somerville, NJ, USA) and polyvinylpyrrolidone (PVP) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were of reagent or HPLC grade and were used without further purification.

2.2. Animal surgery

Nude mice bearing human melanoma [3] were used for the study. To enhance the solubility of betulinic acid in normal saline, PVP and betulinic acid (4:1, w/w) were separately dissolved in methanol and then mixed together. After evaporating the solvent, betulinic acid in PVP was dissolved in normal saline as a suspension solution (25 mg/ml) for i.p. injection. Betulinic acid was administered (500 mg/kg, i.p.) and blood, tumor, lung, liver and kidney were collected after 24 h. Blood was collected through orbital bleeding via heparanized capillary tubes and then stored at −20°C. Dissected tissues were weighed and stored at −20°C until analysis.

2.3. Preparation of stock and standard solutions

A stock solution of betulinic acid was prepared in ethanol (1 mg/ml) and appropriate dilutions were made with ethanol. Standard solutions of betulinic acid in tissue homogenate (0.1–1.0 g of tissue was homogenized with 1 ml of distilled deionized water using a tissue homogenizer, Brinkmann PT 3000, Kinematica AG, Switzerland) were prepared by spiking with an appropriate volume (less than 10 μl) of the diluted stock solution, giving final concentrations of 350, 210, 100, 10 μg/ml (calibration curve 1 for high concentration samples) or 10, 4.7, 1, 0.1 μg/ml (calibration curve 2 for low concentration samples). The samples were then treated as described below and used for the generation of standard calibration curves.

2.4. Sample preparation

Acetonitrile:ethanol (1:1, v/v) was added to each blood and tissue homogenate for deproteinization (2.5 volumes, v/w). After vortex-mixing and centrifugation at 9000 *g* for 2 min, the supernatant was filtered through a 0.22 μm membrane, and 5 μl aliquots of the filtrates were injected directly onto the HPLC column. For blood samples, 3 volumes of distilled deionized water were added before the addition of acetonitrile:ethanol to facilitate the hemolysis of red blood cells and to increase the reproducibility of betulinic acid analysis [8,9].

2.5. Recovery

A recovery test was carried out by comparing peak areas obtained from blood and tissue samples added with a suitable amount stock betulinic acid solution with the corresponding of non-treated standards in ethanol. %Recovery=[peak area (extracted sample)/peak area (stock sample)]×100. Recovery tests for betulinic acid in the biological samples were made at 10 and 1 μg/ml for tumor, liver, lung and kidney homogenates, and 4.7 μg/ml for blood.

2.6. Accuracy and precision

Inter-day and intra-day variability studies were performed by spiking drug-free samples with 1.0 and 10.0 μg/ml betulinic acid. Four samples at each concentration were extracted and analyzed on three consecutive days.

2.7. HPLC–electrospray MS apparatus

The HPLC–electrospray MS system used for the analysis of betulinic acid consisted of a Hewlett Packard (Wilmington, DE, USA) 5989B mass spectrometer, a 59987A electrospray interface, a Hitachi system (Hitachi, Japan) with a L-7100 pump, a L-7400 UV detector and a L-7250 programmable autosampler. The quadrupole temperature of the mass spectrometer was 120°C and the chromatograms were acquired in the SIM (selected ion monitoring) mode for the pseudo-molecular negative ion $[M\text{-}H]^-$ of betulinic acid at m/z 455. The temperature of the drying N_2 gas was 300°C and the flow-rate of nebulizing N_2 was 40 ml/min and maintained at 80 p.s.i. for the N_2 stream in the ion chamber.

Ions were accelerated from the electrospray ion source into the mass spectrometer and focused through hexapole and skimmers. Voltage applied for these electrical elements were optimized while constantly infusing betulinic acid standard solution into the electrospray ion source. The entrance lens and capillary exit voltage were set at 47 and −180 V, respectively. These entrance lens and capillary exit voltages were also optimized by monitoring the ion abundance of direct flow injection of betulinic acid. For the collision-induced dissociation (CID) conditions, the voltages for the capillary exit and the first skimmer were set at −120 and −26 V, respectively.

The HPLC separation was performed with a Waters Novapak C_{18} reversed-phase column (150× 3.9 mm, I.D.) with 5 μm packing material. The mobile phase, 80% acetonitrile, was run at a flow-rate of 0.7 ml/min, and the column effluent was mixed with 0.2% triethylamine (methanol:water: triethylamine, 90:9.8:0.2; flow-rate, 0.13 ml/min) solution to enhance the ionization of the compound in the MS ion chamber.

3. Results and discussion

In order to develop a HPLC–electrospray MS (LC–MS) method for betulinic acid quantification in biological samples, flow injection analyses were performed to determine suitable electrospray parameters. Both positive and negative electrospray ionization conditions were applied for betulinic acid with various acids and/or bases in ethanol. It was found that only negative ion electrospray mass spectra could be obtained during flow-injection analyses. Fig. 2 shows mass spectra of betulinic acid (MW 456) in the negative ion mode (A) and in the CID mode (B). In the negative electrospray ionization mode (Fig. 2A), the deprotonated molecular ion $[M-H]^-$ of betulinic acid at m/z 455 was observed with strong intensity, and no other ions such as sodium-adducts were observed. In the CID mode, an additional ion at m/z 299 $[M\text{-}C_9H_{12}O_2\text{-}H]^-$ was observed. However, sodium-adduct ions were not observed in either positive or negative ion mode even when betulinic acid was prepared in a solution containing 50 μ*M* sodium acetate. In the positive ion electrospray ionization mode, neither protonated ion $[M+H]^+$ nor any adducts were detected. Since betulinic acid has a strong proton donor (carboxyl group) in its structure, the deprotonated ion at m/z 455 can be generated more easily than protonated species.

Fig. 3 shows selected ion chromatograms of drug-free mouse tumor (A); mouse tumor spiked with 0.05 μg/ml of betulinic acid (B); and tumor collected 24 h after i.p. administration of 500 mg/kg of betulinic acid (C). Under the chromatographic conditions used

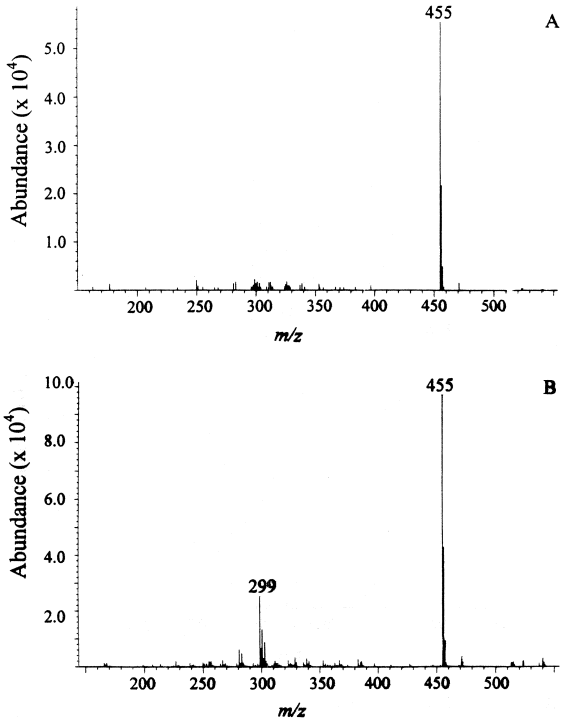

Fig. 2. Mass spectra of betulinic acid in the negative ion mode (A) and in the collision-induced dissociation (CID) mode (B).

above, drug-free tumor samples were free of endogenous materials eluting with the same retention times as betulinic acid, as were the other samples. The retention time for betulinic acid was approximately 6.0 min. Calibration curves were linear from 350 to 10 μg/ml for high concentration samples (curve 1),

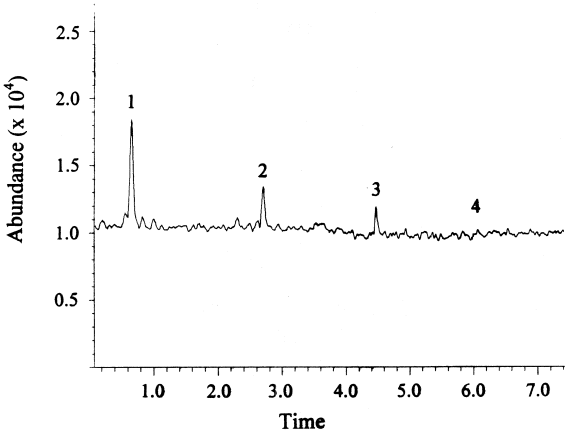

Fig. 4. Limit of detection of betulinic acid $[M-H]^-$ at m/z 455 using negative ion electrospray mass spectrometry with selected ion monitoring (SIM) (5 μl injection, $S/N=3$). 1: 35 pg; 2: 7 pg; 3: 1.4 pg; 4: 0.28 pg.

and from 10 to 0.1 μg/ml for low concentration samples (curve 2). The slopes and intercepts (mean±SD) were calculated as 213 210±19 510 and 3 393 473±271 477 for curve 1, and 423 636±21 181 and 73 155±8778 for curve 2, respectively, with correlation coefficients greater than 0.999. The recoveries of betulinic acid in biological samples were greater than 93%.

To determine the detection limit of betulinic acid, serial dilutions of betulinic acid in ethanol were prepared, injected and monitored at m/z 455. The limit of detection of electrospray LC–MS for betulinic acid was approximately 1.4 pg (0.28 ng/ml, 5 μl injection) (Fig. 4), based on a signal-to-noise

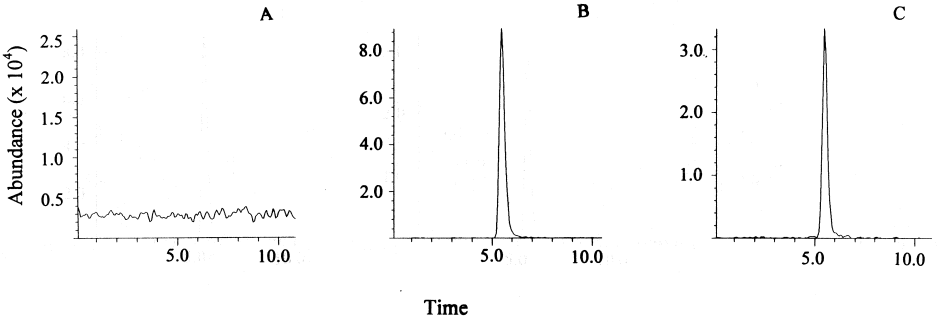

Fig. 3. Representative chromatograms of mouse tumor samples monitored at m/z 455; A=drug-free mouse tumor; B=mouse tumor spiked with 0.05 μg/ml of betulinic acid; C=mouse tumor obtained 24 h after administration of betulinic acid (500 mg/kg, i.p.).

ratio of 3, and the limit of quantitation in this analysis was approximately 25 pg (0.005 μg/ml, 5 μl injection). The mean intra-day coefficient of variation of betulinic acid in these samples was 6.4% (range 3.5–9.1%), whereas the mean inter-day coefficient of variation for analysis of the same samples on three consecutive days was 6.7% (range 4.6–9.0%) (Tables 1 and 2).

Finally, the distribution of betulinic acid in mouse blood, tumor, liver, kidney and lung homogenates was determined by this method, and the results are summarized in Table 3. Clearly, i.p. administration of PVP–betulinic acid complex resulted in the distribution of betulinic acid throughout the body. Interestingly, tumor showed the highest concentration, which was nearly twice the level observed in liver. This factor may help to explain the manner in which betulinic acid can inhibit tumor growth without apparent toxicity [3]. We surmise HPLC–electrospray MS analysis of betulinic acid, as described herein, should be useful for preclinical or clinical evaluations of this potential antitumor agent.

Table 3
Distribution of betulinic acid in mouse blood, tumor, liver, lung and kidney 24 h after i.p. administration of 500 mg/kg (mean±SD, $n=4$)

Sample	Weight (g)	Concentration[a]
Blood[a]	–	1.8±0.5
Tumor	1.04±0.190	452.2±261.2
Liver	1.21±0.100	223.9±80.3
Lung	0.40±0.037	74.8±63.7
Kidney	0.36±0.029	95.8±122.8

[a] Concentration units for blood=μg/ml; all other values are μg/g.

Table 1
Intra-day assay accuracy and precision of betulinic acid

Sample	Added (μg/ml)	Mean observed concentration (μg/ml)	Accuracy (%)	C.V.[a] (%)
Blood	4.7	4.96	105.5	9.1
Tumor	10.0	9.62	96.2	3.5
	1.0	1.04	104.0	7.7
Liver	10.0	9.71	97.1	5.9
	1.0	0.92	91.8	6.3
Lung	10.0	9.82	98.2	4.1
	1.0	1.06	106.3	7.9
Kidney	10.0	9.67	96.7	3.9
	1.0	1.05	105.1	8.8

[a] Coefficient of variation (C.V.)=(standard deviation/mean)×100.

Table 2
Inter-day assay accuracy and precision of betulinic acid

Sample	Added (μg/ml)	Mean observed concentration (μg/ml)	Accuracy (%)	C.V.[a] (%)
Blood	4.7	5.09	108.3	5.3
Tumor	10.0	9.36	93.6	8.7
	1.0	0.96	96.2	9.0
Liver	10.0	9.50	95.0	5.5
	1.0	1.06	105.9	4.6
Lung	10.0	9.79	97.9	6.2
	1.0	1.04	103.7	8.9
Kidney	10.0	9.34	93.4	5.0
	1.0	1.08	108.1	7.1

[a] Coefficient of variation (C.V.)=(standard deviation/mean)×100.

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